

A possible Involvement of Prostaglandin E₂ in the Reproduction of Female Crested Newt, *Triturus carnifex*

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ABSTRACT—Plasma prostaglandin E₂ (PGE₂), prostaglandin F_{2α} (PGF_{2α}), and sex steroid hormones (progesterone, androgens, and 17β-estradiol) were determined in the female crested newt, *Triturus carnifex*, during the annual reproductive cycle. *In vivo* experiments were carried out to study the effects of PGE₂ and PGF_{2α} on plasma sex steroid hormones during prereproduction, reproduction, and postreproduction; simultaneously, *in vitro* experiments were performed to study the effects of these two prostaglandins on sex steroid hormones ovarian release. The effects of one week's captivity on PGE₂, PGF_{2α} and sex steroid hormones were also evaluated. The PGE₂ plasma level was low from October to December, then it rapidly increased to peak in March, after which it soon fell to reach its minimum value in May. Plasma PGF_{2α} and sex steroid hormones showed similar trends to those found in previous studies. From December to April, the PGE₂ plasma values were negatively correlated to those of PGF_{2α} and positively to those of androgens; PGF_{2α} plasma values were positively correlated to those of estradiol. PGE₂ *in vivo* treatment increased plasma progesterone and decreased 17β-estradiol in April, while PGF_{2α} induced the opposite effects in the same month; PGE₂ increased androgens in January and March, and PGF_{2α} increased androgens in April. The *in vitro* experiments were in agreement. These results suggest that PGE₂ and PGF_{2α} play opposite role(s) in the reproductive processes of the female *Triturus carnifex*. PGE₂ could be involved in the reproduction processes through androgen secretion, while PGF_{2α} in the ending of reproduction through estradiol increase.

INTRODUCTION

In mammals, prostaglandins (PGs) of both F and E series intervene in the regulation of the ovary functions, including steroidogenesis [1–4]. PGs are involved in ovulation of the chicken follicle [5] and in inducing the relaxation of the avian oviduct [6]. PGs are also implied in the regulation of reproduction in reptiles [7–10]. There is little information, however, on the role of PGs in amphibian reproductive processes. In the anuran, *Rana esculenta* [11, 12], and in the urodele, *Triturus carnifex* [13, 14], plasma prostaglandin F_{2α} (PGF_{2α}) levels change in relation to the various periods of the annual reproductive cycle, and in both species PGF_{2α} could be implied in breeding period termination.

A recent *in vitro* study, carried out on the male

Triturus carnifex abdominal gland, suggested for PGE₂ a role in inducing pheromonal activity [15]; these data indicated an involvement of this prostaglandin in reproductive processes.

It seemed, therefore, of interest to determine the annual profile of PGE₂, in relation to that of PGF_{2α} and of sex steroid hormones in the female crested newt, *Triturus carnifex*, and, in addition, to evaluate the PGE₂ and PGF_{2α} effects on plasma levels and ovarian output of sex steroid hormones.

MATERIALS AND METHODS

Animals

The reproductive cycle of the *Triturus carnifex* population comprises two major phases: during the summer period the animals disappear underground (August–September), during the other months the newts are in the pond and undertake reproduction during the cold months (January to

March).

Adult female newts, *Triturus carnifex* (Laur.), were collected (15 each month) in a small mountain pond (Colfiorito, Umbria, Italy; 870 m above sea level), from October to July during 1990/91. In that place, newts hide on land during August and September and therefore, in these two months, it was not possible to obtain enough animals for our study. At once, after capture, newts were anesthetized in the field with 3-aminobenzoic acid ethyl ester (Tricaine, Sigma, USA) and bled through a heparinized microtube inserted in the heart. Individual blood samples were collected into chilled tubes containing acetylsalicylic acid (Aspirin, Sigma) and EDTA (5 μ g and 7 μ g/ml of blood, respectively) [11]. After centrifugation, plasma samples were kept at -20°C until use.

Captivity

In December (prereproduction), January (beginning of reproduction), March (ending of reproduction), and April (postreproduction), 15 female newts were captured and bled in the field as reported above (control I), while simultaneously 15 other animals were transferred to our laboratory aquaria, kept under natural photothermal conditions and fed on larvae of Chironomidae, *Tubifex* and *Daphnia*, *ad libitum* to study the effects of captivity on plasma hormone levels. A week later, the animals in captivity were bled, and simultaneously yet another 15 were sacrificed in the field (control II).

In vivo experiment

In December, January, March, and April, 84 female newts (for each month) were captured, transferred to our laboratory and kept under natural photothermal conditions. One week later, the animals were divided into 4 groups: a) 21 newts received a single s.c. injection of 350 ng PGE_2 (Sigma; ca 35 ng/g body weight) dissolved in 100 μl amphibian saline (0.64% w/v NaCl solution); b) 21 newts received a single s.c. injection of 300 ng $\text{PGF}_{2\alpha}$ (Sigma; ca 30 ng/g body weight) dissolved in 100 μl amphibian saline; c) 21 newts received a single s.c. injection of 100 μl amphibian saline only; d) 21 newts were untreated. Seven of the 21

animals of each of the groups were bled, as described above, 6, 24, and 48 hr after treatment. After centrifugation, plasma samples were kept at -20°C until use. A further batch of seven untreated animals were bled at the beginning of the experiments (controls, time 0). The times of treatment and the minimum effective doses of PGE_2 and $\text{PGF}_{2\alpha}$ were chosen after preliminary tests (data not shown).

In vitro experiment

The method used for the *in vitro* experiment followed Gobbetti and Zerani [15, 16]. In December, January, March, and April, 7 female newts (for each month) were captured, transferred to laboratory aquaria, and maintained as reported above. One week later, the animals were decapitated, the ovaries rapidly removed and placed in cold Dulbecco's modified Eagle medium (DME; Sigma) containing 10 mM Hepes, 1 mg Penicillin G/ml, and 2 mg streptomycin/ml. For each month, the ovary from one animal was divided into equally sized fragments, pooled and equally distributed over 12 incubation wells. Multiwell tissue culture plates (Becton Dickinson, USA) were utilized. Each incubation set of wells was divided into 3 experimental groups (each consisting of 4 wells). The experimental groups were: a) ovarian tissue incubated with DME alone; b) ovarian tissue incubated with DME plus PGE_2 (50 ng); c) ovarian tissue incubated with DME plus $\text{PGF}_{2\alpha}$ (50 ng). The final volume of each well was 1 ml. Culture plates were wrapped with aluminium foil and incubated in a shaking water bath (19°C), set at 30 revolutions/min. One well of each experimental group was removed, respectively, after 30, 60, 120 and 240 min of incubation. The incubation medium samples were immediately stored at -20°C for later hormone determination; ovarian tissues were soon homogenized in amphibian saline, and protein content was determined by the Protein Assay method (Bio-Rad, USA). In addition, the whole experiment was repeated with incubation sets without ovarian tissue. The experiment was replicated 7 times for each month. Preliminary evidence led to our choice for the incubation conditions and the minimum effective doses of PGE_2 and $\text{PGF}_{2\alpha}$ utilized in the present *in*

vitro study (data not shown).

PGE₂, PGF_{2α}, progesterone, androgens, and 17β-estradiol determination

PGE₂ was determined following the radioimmunochemical method (RIA) for plasma [13, 14] and incubation media [15], PGF_{2α} determinations used for this species are here briefly described. PGE₂ determinations were carried out on duplicate plasma samples (100 μl) and incubation media (500 μl) that were extracted with 10 volumes of diethyl ether for 5 min. The mixtures were briefly centrifuged, organic fractions were transferred into glass tubes and evaporated to dryness under a nitrogen stream. The extracts were resuspended with 100 μl of assay buffer and assayed. The recovery of added labelled PGE₂ was $85.9 \pm 0.84\%$. The parallelism among the standard curve in buffer, a standard curve in incubation medium (then extracted), and a serial dilution of a single incubation medium sample (extracted) were constant.

The progesterone, androgens, and 17β-estradiol content of the plasma and incubation media was determined by RIA according to the previously reported methods [13, 15, 17].

The following sensitivities were recorded: PGE₂, 18 pg (intra-assay variability: 6.5%; inter-assay variability: 12%); PGF_{2α}, 17 pg (intra-assay variability: 5%; inter-assay variability: 11.5%); progesterone, 7.0 pg (intra-assay variability: 5%; inter-assay variability: 11.5%); androgens, 9.0 pg (intra-assay variability: 6%; inter-assay variability: 9%); 17β-estradiol, 8.5 pg (intra-assay variability: 4%; inter-assay variability: 9%). The PGF_{2α}, progesterone, testosterone, and 17β-estradiol antisera were provided by Dr. G. F. Bolelli and Dr. F. Franceschetti (CNR-Physiopathology of Reproduction Service, University of Bologna, Italy), the PGE₂ antiserum was purchased from Cayman Chemical (USA). Testosterone was not separated from 5α-dihydrotestosterone and therefore, since the antiserum used is not specific, the data are expressed as androgens. Tritiated PGE₂, PGF_{2α}, progesterone, testosterone, and 17β-estradiol were purchased from Amersham International (England), non-radioactive PGE₂, progesterone, testosterone, and 17β-estradiol from Sigma.

Statistics

Data relative to each hormone were submitted to analysis of variance (ANOVA) followed by Duncan's multiple range test [18, 19]. Correlation coefficients followed Scossiroli and Palenzona [20].

RESULTS

Hormone annual reproductive cycles

The PGE₂ plasma level was low from October to December, then it rapidly increased to peak in March ($P < 0.01$ vs all months) and soon fell to reach its minimum value in May ($P < 0.01$ vs January, February, March, April, June, and July), then PGE₂ level increased during the summer and peaked in July ($P < 0.01$ vs all months except February) (Fig. 1). The PGF_{2α} plasma level in-

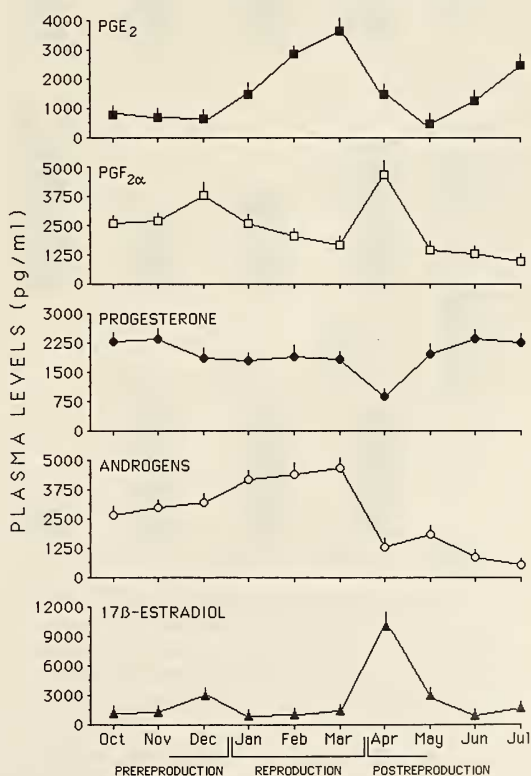


FIG. 1. Plasma levels of PGE₂, PGF_{2α}, and sex steroid hormones during the annual reproductive cycle in female crested newt, *Triturus carnifex*. Each mean refers to 15 determinations $\pm$ S.D..

creased in autumn to peak in December ($P < 0.01$ vs all months except April), decreased from January to March and peaked again in April ($P < 0.01$ vs all months except December), and was low from May to July (Fig. 1). The progesterone plasma level exhibited minor changes, reaching the minimum value in April ($P < 0.01$ vs all months) (Fig. 1). The plasma androgens increased from October to December and more from January to March ($P < 0.01$ vs October, November, December, April, May, June, and July), they started to drop in April and reached the minimum value in July ($P < 0.01$ vs all months except June) (Fig. 1). The 17β -

estradiol plasma level peaked in December ($P < 0.01$ vs all months except April, May, and July) and in April ($P < 0.01$ vs all months) (Fig. 1). From December to April the following correlations were found: PGE_2 plasma values were negatively correlated to those of $PGF_{2\alpha}$ ($r = -0.523$; $df = 73$; $P < 0.001$) and estradiol ($r = -0.469$; $df = 73$; $P < 0.001$), and positively correlated to those of androgens ($r = 0.501$; $df = 73$; $P < 0.001$), $PGF_{2\alpha}$ plasma values were negatively correlated to those of androgens ($r = -0.545$; $df = 73$; $P < 0.001$), and positively to those of estradiol ($r = 0.434$; $df = 73$; $P < 0.001$), androgens plasma values were negatively correlated to those of estradiol ($r = -0.408$; $df = 73$; $P < 0.001$).

Captivity

One week's captivity in laboratory aquaria decreased PGE_2 plasma levels in January and March ($P < 0.01$), $PGF_{2\alpha}$ in April ($P < 0.01$) and androgens in January, March and April ($P < 0.01$) (Fig. 2).

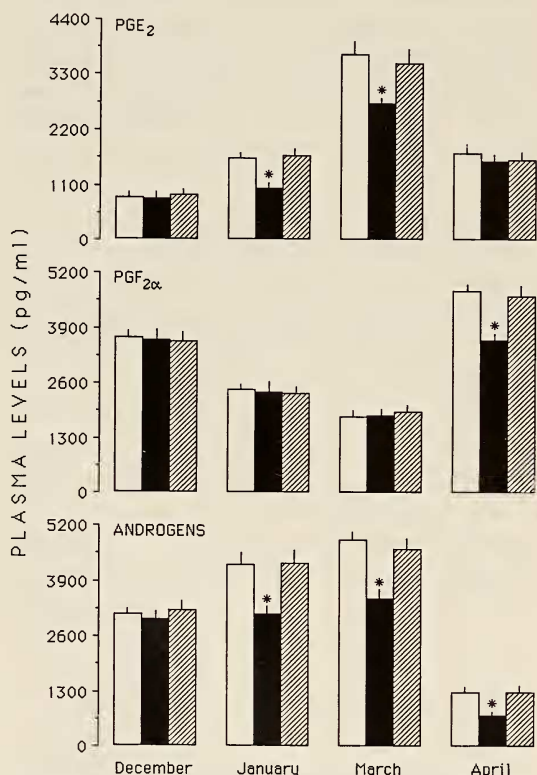


FIG. 2. Effects of one week's captivity on PGE_2 , $PGF_{2\alpha}$, and androgens plasma levels in female crested newt, *Triturus cristatus*, during December (prereproduction), January (reproduction beginning), March (reproduction ending), and April (postreproduction). Experimental groups: (□): newts captured in the field and bled at once (control I); (■): newts bled after one week's captivity in laboratory; (▨): newts captured and bled at once in the field, one week after control I (control II). Each mean refers to 15 determinations $\pm$ S.D.. * $P < 0.01$ vs control I and II (Duncan's multiple range test).

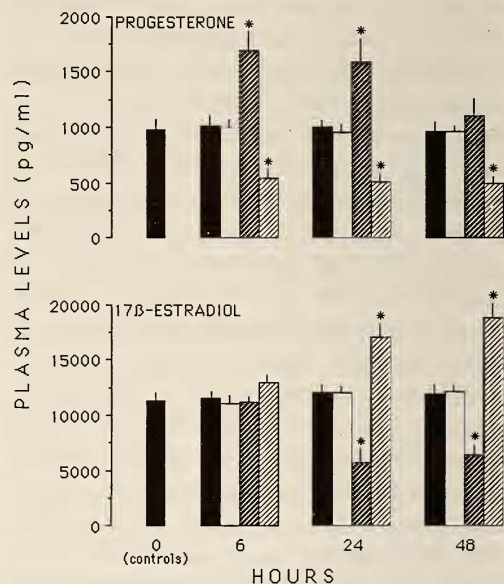


FIG. 3. *In vivo* effects of 350 ng PGE_2 or 300 ng $PGF_{2\alpha}$ injection on progesterone and 17β -estradiol plasma levels in female crested newt, *Triturus cristatus*, during April (postreproduction). Experimental groups: (■): untreated newts; (□): amphibian-saline-only injected newts; (▨): PGE_2 injected newts; (▩): $PGF_{2\alpha}$ injected newts. Each mean refers to 7 determinations $\pm$ S.D.. * $P < 0.01$ vs untreated and amphibian-saline-only injected newts (Duncan's multiple range test).

In vivo experiment

PGE₂ treatment increased progesterone level in April at 6 and 24 hr ($P < 0.01$) (Fig. 3), androgens in January and March at 6, 24 and 48 hr ($P < 0.01$) (Fig. 4), and decreased 17 β -estradiol in April at 24 and 48 hr ($P < 0.01$) (Fig. 3).

PGF_{2 α} treatment decreased progesterone level in April at 6, 24 and 48 hr ($P < 0.01$) (Fig. 3), increased androgens in April at 24 and 48 hr ($P < 0.01$) (Fig. 4), and 17 β -estradiol in April at 24 and 48 hr ($P < 0.01$) (Fig. 3).

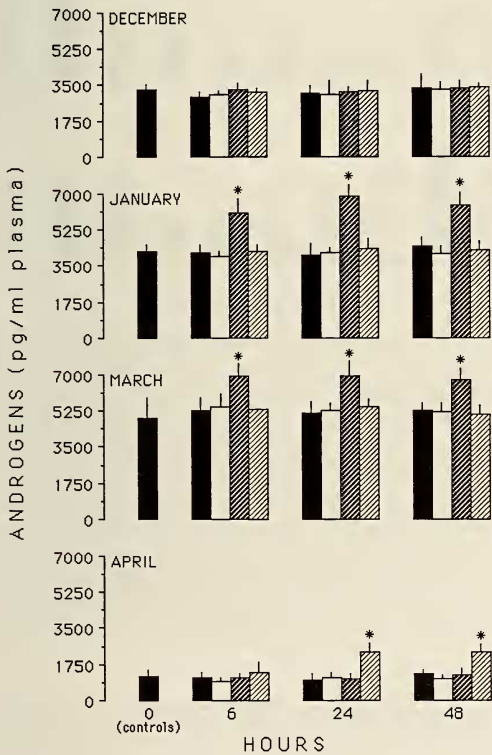


FIG. 4. *In vivo* effects of 350 ng PGE₂ or 300 ng PGF_{2 α} injection on androgens plasma levels in female crested newt, *Triturus carnifex*, during December (prereproduction), January (reproduction beginning), March (reproduction ending), and April (postreproduction). Experimental groups: (■): untreated newts; (▨): amphibian-saline-only injected newts; (▩): PGE₂ injected newts; (▨): PGF_{2 α} injected newts. Each mean refers to 7 determinations $\pm$ S.D.. * $P < 0.01$ vs untreated and amphibian-saline-only injected newts (Duncan's multiple range test).

In vitro experiment

PGE₂ basal release was higher in January and March ($P < 0.01$) compared with December and April; March values were higher ($P < 0.01$) than those of January (Fig. 5). PGF_{2 α} basal release was higher in April ($P < 0.01$) compared with the other months (Fig. 5). Progesterone basal release was lower in April ($P < 0.01$) compared with the other months (Fig. 6). Basal release of androgens was higher in January and March ($P < 0.01$) compared with the other months (Fig. 7). Estradiol basal level was higher in December and April ($P < 0.01$); April values were higher ($P < 0.01$) than those of December (Fig. 8). At all incubation times, the

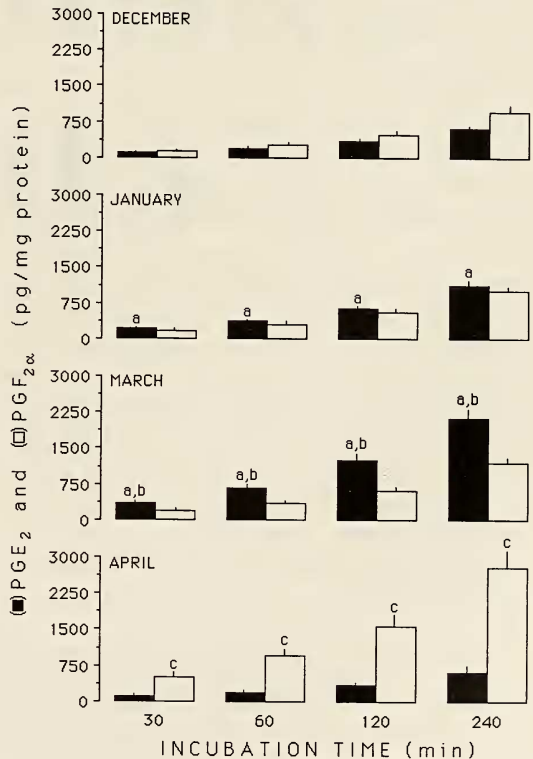


FIG. 5. *In vitro* basal release of PGE₂ (■) and PGF_{2 α} (□) from ovary of female crested newt, *Triturus carnifex*, incubated in December (prereproduction), January (reproduction beginning), March (reproduction ending), and April (postreproduction). Each mean refers to 7 determinations $\pm$ S.D.. a, $P < 0.01$ vs same time December and April; b, $P < 0.01$ vs same time January; c, $P < 0.01$ vs same time December, January, and March (Duncan's multiple range test).

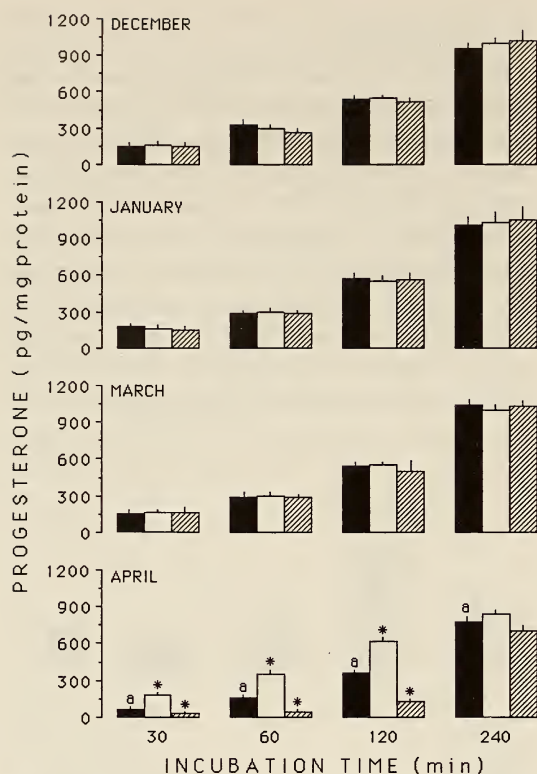


FIG. 6. *In vitro* effects of 50 ng PGE₂ or 50 ng PGE_{2α} on progesterone release from ovary of female crested newt, *Triturus cristatus*, incubated in December (prereproduction), January (reproduction beginning), March (reproduction ending), and April (postreproduction). Experimental groups: (■): ovary incubated with medium alone; (□): ovary incubated with PGE₂; (▨): ovary incubated with PGF_{2α}. Each mean refers to 7 determinations $\pm$ S.D.. a, $P < 0.01$ vs same time December, January, and March; * $P < 0.01$ vs same time medium-alone (Duncan's multiple range test).

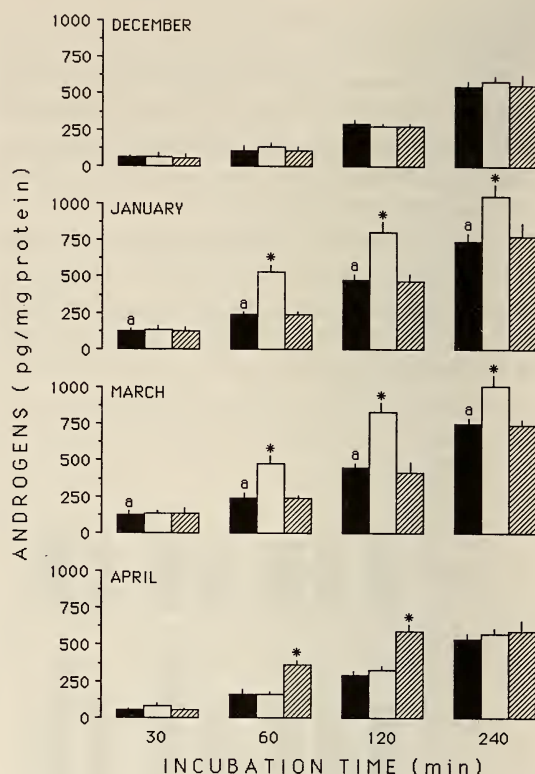


FIG. 7. *In vitro* effects of 50 ng PGE₂ or 50 ng PGF_{2α} on androgens release from ovary of female crested newt, *Triturus cristatus*, incubated in December (prereproduction), January (reproduction beginning), March (reproduction ending), and April (postreproduction). Experimental groups: (■): ovary incubated with medium alone; (□): ovary incubated with PGE₂; (▨): ovary incubated with PGF_{2α}. Each mean refers to 7 determinations $\pm$ S.D.. a, $P < 0.01$ vs same time December and April; * $P < 0.01$ vs same time medium-alone (Duncan's multiple range test).

TABLE 1. Correlation coefficients among PGE₂, PGF_{2α}, androgens (A), and 17 β -estradiol (E) levels released by female *Triturus cristatus* ovary incubated at various times during December, January, March, and April

	30 min	Incubation times		240 min
		60 min	120 min	
PGE ₂ vs PGF _{2α}	-0.768	-0.657	-0.743	-0.757
PGF _{2α} vs A	0.724	0.745	0.774	0.761
PGE ₂ vs E	-0.650	-0.637	-0.672	-0.621
PGF _{2α} vs A	-0.733	-0.759	-0.731	-0.749
PGF _{2α} vs E	0.715	0.748	0.686	0.668
A vs E	-0.620	-0.670	-0.646	-0.645

All correlations show the same level of significance ($P < 0.001$). df=26.

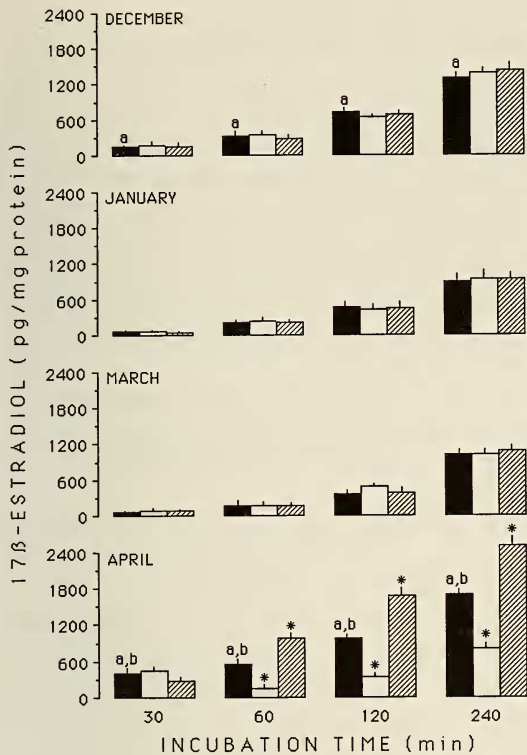


FIG. 8. *In vitro* effects of 50 ng PGE₂ or 50 ng PGF_{2α} on 17β-estradiol release from ovary of female crested newt, *Triturus carnifex*, incubated in December (prereproduction), January (reproduction beginning), March (reproduction ending), and April (postreproduction). Experimental groups: (■): ovary incubated with medium alone; (□): ovary incubated with PGE₂; (▨): ovary incubated with PGF_{2α}. Each mean refers to 7 determinations $\pm$ S.D.. a, $P < 0.01$ vs same time January and March; b, $P < 0.01$ vs same time December; * $P < 0.01$ vs same time medium-alone (Duncan's multiple range test).

PGE₂ values were negatively correlated to those of PGF_{2α} ($P < 0.01$) and estradiol ($P < 0.001$), and positively correlated to those of androgens ($P < 0.001$); PGF_{2α} values were negatively correlated to those of androgens ($P < 0.001$), and positively to those of estradiol ($P < 0.001$); androgens values were negatively correlated to those of estradiol ($P < 0.001$) (Tab. 1).

PGE₂ treatment increased progesterone release in April at 30, 60 and 120 min ($P < 0.01$) (Fig. 6), androgens in January and March at 60, 120 and 240 min ($P < 0.01$) (Fig. 7), and decreased estradiol in

April at 60, 120 and 240 min ($P < 0.01$) (Fig. 8). PGF_{2α} treatment decreased progesterone levels in April at 30, 60 and 120 min ($P < 0.01$) (Fig. 6), increased androgens in April at 60 and 120 min ($P < 0.01$) (Fig. 7), and estradiol in April at 60, 120 and 240 min ($P < 0.01$) (Fig. 8).

DISCUSSION

The mountain population of female *Triturus carnifex*, utilized in this study, shows a discontinuous reproductive cycle similar to those described in several other newts living in temperate zones [21–23]. In this female newt, ovulations and egg depositions occur from January to the end of March, the oogenetic cycle in spring and summer, and vitellogenesis in autumn and winter [24].

This is the first work to evidence *in vivo* and *in vitro* the presence of PGE₂ in female *Triturus carnifex*. The PGE₂ plasma titers showed peculiar changes during the newt annual reproductive cycle; during the reproductive period (January–March), this prostaglandin had high values which rapidly fell during postreproduction (April). The high plasma levels of PGE₂ during reproduction suggest the involvement of this prostaglandin in the breeding processes. PGF_{2α}, progesterone, androgens and estradiol plasma levels showed similar annual trends to those reported in previous studies [14, 24]. Briefly, the April increase of plasma PGF_{2α} was simultaneous with an estradiol rise and a progesterone drop. High plasma values of androgens characterized the reproductive period in female *Triturus carnifex*, confirming previous studies carried out in the same species [25, 26] and in the same population [24], which established the role of androgens in inducing the reproductive processes was addressed in the female crested newt. In this context we recall that, in another amphibian species, *Rana esculenta*, the highest values of male and female brain androgen receptors were found during the reproductive period [27]. As regards estradiol plasma pattern, low levels were found in autumn and winter, when vitellogenesis occurs. It was well established in amphibians that estradiol induces vitellogenin synthesis [28], therefore the low estradiol plasma values found during vitellogenesis are difficult to

explain, but, in this context, we recall that similar data were obtained in other amphibian species [29]. In accordance with the plasma values, the *in vitro* studies demonstrated that ovarian tissue released high amounts of PGE₂ and androgens during reproduction and high levels of PGF_{2α} and estradiol during postreproduction, while in this latter period progesterone was low.

The *in vivo* and *in vitro* PGE₂ treatment increased androgens levels during reproduction suggesting a causal relationship between these two hormones. This hypothesis is also supported by the positive correlation between PGE₂ and androgens found in the plasma of the animals sacrificed in the field. The same positive correlation was also found between the basal values of PGE₂ and androgens obtained in the *in vitro* experiment. These results seem further to indicate that PGE₂ is involved in the reproductive processes, by the regulation of the androgens synthesis, even if other mechanisms cannot be excluded. The involvement of this prostaglandin in reproduction is also suggested by previous studies which attribute to PGE₂ a role in inducing pheromonal activity by the abdominal gland of *Triturus carnifex* [15]. Also in another amphibian species, *Xenopus laevis*, PGE₂ was found to be involved in the breeding processes, since this prostaglandin induced the sexual behavior [30].

The *in vivo* and *in vitro* results indicated that PGE₂ treatment in animals captured in April induced opposite effects to those determined by PGF_{2α} administered in the same month. In fact, PGE₂ *in vivo* and *in vitro* increased progesterone and decreased estradiol, while PGF_{2α} decreased progesterone and increased estradiol during this period, in agreement with the plasmatic trends here and previously reported [14, 24]. The causal relationship which binds the PGF_{2α} and estradiol plasma patterns supported the hypothesis that PGF_{2α} contributed to reproductive period termination. In fact, in several temperate-zone living amphibians and reptiles, a significant estradiol plasma rise occurred near the end of reproduction [31–33]. This steroid is believed responsible for the breeding interruption probably through a negative feedback mechanism at local [34] and central [35] levels.

Taken together, these results suggest that PGE₂ and PGF_{2α} play opposite role(s) in the reproductive processes of the female *Triturus carnifex*. Summarizing, PGE₂ is involved in the reproduction through androgens secretion, while PGF_{2α} in the ending of reproduction through estradiol increase. This possible antagonistic role between PGE₂ and PGF_{2α} is supported by their negative correlation found in the plasma. This hypothesis is in agreement with previous studies which assign an inhibitory role to PGF_{2α} in sexual behavior in the reptiles, *Anolis carolinensis* and *Thamnophis sirtalis parietalis* [36, 37] and in the amphibian, *Bufo americanus* [38], but a stimulatory role to PGE₂ in receptivity behavior in another amphibian, *Xenopus laevis* [30].

The antagonistic effect of PGE₂ and PGF_{2α} in the regulation of *in vivo* and *in vitro* progesterone release found during postreproduction is still unknown. We recall that a positive 3β-hydroxy-steroid dehydrogenase response was reported in the postovulatory structures of *Triturus carnifex* [39], *Rana esculenta* [40] and *Rana cyanophlyctis* [41], but evidence of ultrastructural analysis is lacking [22]. Nevertheless, in mammals, PGE₂ counteracts the luteolytic effect of PGF_{2α} and might be one of several factors prolonging the life span of the corpus luteum [42, 43].

Finally, this work confirms that captivity caused a decrease of circulating PGs and androgens. These results are in agreement with those reported for this [14] and other amphibian species [44–47].

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